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Molecular characterization of *Toxocara* spp. from soil of public areas in Ahvaz southwestern Iran

Shahram Khademvatan[a,b,*], Rahman Abdizadeh[b], Mahdi Tavalla[b]

[a] Research Institute for Infectious Diseases of Digestive System, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[b] Department of Medical Parasitology, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

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ABSTRACT

In the present study, the microscopy and polymerase chain reaction methods were used for detection and identification of soil contamination by *Toxocara* eggs in squares, streets, public parks, and rubbish dumps in Ahvaz, southwestern Iran.

A total of 210 soil samples were collected from different parts of the city and examined by microscopy and polymerase chain reaction (PCR) methods, following sodium nitrate flotation. Nucleotide sequencing was performed to confirm the results of the PCR method. *Toxocara* eggs were found in 64 and 71 soil samples using the microscopy and PCR methods, respectively. The highest contamination rate was observed in the central part of Ahvaz (39.5% and 46.5% by the microscopy and PCR methods, respectively). Based on internal transcribed spacer 2 (ITS2) PCR identification, 28% of the samples were diagnosed as *Toxocara cati* and 5.7% as *Toxocara canis*; no mixed contamination was observed. DNA sequencing of the ITS2 gene confirmed our findings.

Compared to the conventional microscopic detection following by flotation, used as the gold standard, the PCR method appears to be rapid and sensitive as well as allows analysis of *Toxocara* spp. isolated from soil independent of the stage of egg development. Therefore, the PCR method appears to be a valuable tool for the diagnosis and differentiation of *Toxocara* spp. from soil samples in epidemiological studies, and will help the local health systems in effective prevention and control of disease.

1. Introduction

Human toxocariasis is a widespread helminthic zoonotic disease that is caused by the larval stage of *Toxocara canis* (dog roundworm) and *Toxocara cati* (cat roundworm) (Despommier, 2003; Fisher, 2003; Lee et al., 2010). Infected cats and dogs pass more than 50,000 eggs/g feces into the environment daily. After 3–6 weeks, depending on the temperature and moisture, eggs become embryonated and infective (Despommier, 2003; Lee et al., 2010). Therefore, these animals play a significant role in the epidemiology of toxocariasis, especially in the tropical, subtropical, and temperate regions of the world (Azian et al., 2008; Fisher, 2003).

Infections of humans are commonly acquired via accidental ingestion of embryonated eggs in the soil and uncooked vegetables (Akao and Ohta, 2007; De Oliveira and Germano, 1992; Hoffmeister et al., 2007; Lee et al., 2010; Szabová et al., 2007; Uga et al., 2009; Yoshikawa et al., 2008; Zibaei et al., 2010). Depending on the number of ingested eggs, localization of the larvae, and host responses, the infection in humans has four clinical manifestations, namely visceral larva migrans (VLM), ocular larva migrant (OLM), covert toxocariasis (CT), and neurological toxocariasis (NLM) (Azian et al., 2008; Congdon and Lloyd, 2011; Macpherson, 2005; Smith et al., 2009).

In Iran, stray animals feast on rubbish and leftover food countless times and discharge helminth eggs and protozoan cysts into public environments (Khademvatan et al., 2013). The increasing number of stray dogs and cats in urban and rural areas, easy access of animals to public parks, streets, rubbish dumps and increasing popularity of keeping animals as pets may contribute to soil contamination and increase parasitic infections, such as toxoplasmosis and toxocariasis (Despommier, 2003).

Toxocara eggs remain viable in the soil for a long time depending on several factors, such as climatic conditions, humidity of the soil, and exposure to sunlight (Storey and Phillips, 1985). Consequently, soil contamination seems to be the most direct

* Corresponding author at: Research Institute for Infectious Diseases of Digestive System and Department of Medical Parasitology, Ahvaz Jundishapur University of Medical Sciences, PO Box: 613715794, Ahvaz, Iran. Tel.: +98 611 3367543x50; fax: +98 611 3332036.
E-mail address: khademvatan@yahoo.com (S. Khademvatan).

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indicator of the risk to human populations, mainly in children. For this reason, many studies have been conducted in recent years to determine the prevalence of *Toxocara* eggs in the soil from parks, playgrounds, sandpits, beaches, backyards, gardens, farmyards, and other urban and rural areas (Akao and Ohta, 2007; Blaszkowska et al., 2011; Motazedian et al., 2006; Ruiz de Ybxkáñez et al., 2001; Zibaei et al., 2010). Determination of *Toxocara* in feces and soil by microscopy after sedimentation and flotation is the most common method. Because eggs of both *Toxocara* species are similar in size and morphology, identification based on morphological features by light and scanning microscopy is not conclusive (Borecka, 2004; Fahrion et al., 2011). Therefore, the polymerase chain reaction (PCR) method, as an alternative method, is extremely sensitive and considered as a specific method to differentiate between the species (Blaszkowska et al., 2011; Borecka and Gawor, 2008; Uga et al., 2000; Zhu et al., 2001).

Since in Iran, few studies have been conducted about *Toxocara* spp. the present study aimed to evaluate the soil contamination of public areas in the city of Ahvaz, southwestern Iran, by *Toxocara* spp. eggs and to use molecular methods for identifying species of *Toxocara* eggs isolated from the soil.

2. Materials and methods

2.1. Study area

This survey was conducted from November 2011 to February 2012 in Ahvaz city, the capital of Khuzestan Province (southwestern Iran, 31°50′ N and 49°11′ E). Ahvaz city is the seventh largest city in Iran, with an area of more than 200 km^2. It has a desert climate with long, extremely hot summers and mild, short winters. Summer temperatures routinely exceed 50 °C while in winter the minimum temperature could fall to approximately 5 °C. The average annual rainfall is approximately 230 mm.

2.2. Collection and recovery of soil samples

The city was divided into five divisions (north, south, east, west and center) all of which are residential areas. A total of 210 soil samples were collected from sidelines of the streets, public parks, squares, and rubbish dumps. The superficial dirt of the soil was eliminated, and approximately 200 g of soil was removed from an approximate depth of 3 cm (Matsuo and Nakashio, 2005). All samples were placed in sealed polyethylene bags, labeled with a number, and stored at 4 °C until processing, which was performed within 24 h.

2.3. Detection of eggs

The soil samples were spread in a tray, dried at room temperature for 1–2 days, and sifted through a 150-μm mesh sieve. All steps for the recovery of *Toxocara* eggs from the samples were used according to a previously described technique (Mizgajska-Wiktor, 2005). In brief, 60 ml of 5% NaOH was poured into 40 g of soil, left for 1 h, shaken for 20 min, and centrifuged at 1500 rpm for 10 min. The supernatant fraction was discarded and centrifugation was repeated with 60 ml of water (1500 rpm for 3 min). The sediment was suspended in 60 ml of saturated flotation fluid ($NaNO_3$) with a specific gravity of 1.30 and then centrifuged (1500 rpm, 10 min). A 24 mm × 24 mm cover slip was placed on the surface and the sample was examined at a magnification of 40× and 100× for *Toxocara* eggs. Embryonation stage of *Toxocara* eggs was detected under light microscope based on morphologic features such as presence of larva inside the eggs.

2.4. DNA extraction and PCR

Genomic DNA from all 210 flotation fluid was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions, with a minor modification. The samples were first subjected to three freeze–thaw cycles, and proteinase K digestion was performed overnight (~16 h) as suggested by Borecka and Gawor (2008). DNA concentration was measured spectrophotometrically at 260 nm. For molecular diagnosis, species-specific primers were chosen from internal transcribed spacer 2 (ITS2) gene sequences that were previously described as *Tcan1* (5′-AGTATGATGGGCGCGCCAAT-3′) and *NC2* (5′-TAGTTTCTTTTCCTCCGCT-3′) for *T. canis*, and *Tcat1* (5′-GGAGAAGTAAACTC-3′) and *NC2* for *T. cati* (Borecka and Gawor, 2008; Wu et al., 1997).

The amplification reactions were performed in 50 μl reaction mixtures containing 250 μM of deoxynucleotide, 100 pmol of each primer, 50 mM KCl, 10 mM-Tris–HCl (pH 9), 3 mM $MgCl_2$, 10% dimethyl sulfoxide (DMSO, Sigma), 2 U of Taq DNA polymerase (Fermentas), and 10–15 ng of template DNA. PCR was performed using a PCR programmed thermocycler (MyCycler; Bio-Rad, Hercules, CA). Amplifications were performed under the following conditions: initial cycle at 94 °C for 30 s, followed by 30 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 30 s and extension by polymerase at 72 °C for 30 s and a final cycle at 72 °C for 5 min. Electrophoresis was performed by adding 5 μl of the PCR products to a 1.8% (w/w) agarose gel and stained with ethidium bromide for 45 min at 100 V. Bands were observed by ultraviolet transillumination.

2.5. Sequencing and phylogenetic analysis

PCR amplification of the ITS2 gene from nine randomly selected samples was sequenced by MWG (Germany), and the resulting data were analyzed using Chromas software (http://www.technelysium.com.au/Chromas.html). The GenBank database was searched for similar sequences using BLAST (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov/BLAST/) and the output was analyzed to find a significant homology.

DNA sequences were aligned with ClustalW (Larkin et al., 2007) and the ITS sequences were entered in PAUP* to generate phylograms for each dataset (Dettman et al., 2006, 2003).

2.6. Statistical analysis

Statistical analysis was performed using PASW Statistics (version 18.0.0.) software. Data were presented as the prevalence of *Toxocara* spp. in the soil ±95% CI Student's *t*-test was used to compare the frequencies of *Toxocara* prevalence between city areas, at a CI of 95%. The overall agreement between the employed diagnostic methods results was calculated using kappa index.

3. Results

This study focused on *Toxocara* spp. contamination in the soil of Ahvaz city (southwest Iran) using the microscopy and PCR methods. The results of the contamination rate in the various divisions of the city are presented in Table 1. With the microscopy method, 64 of 210 soil samples collected from various parts of the city were found to be contaminated with *Toxocara* eggs, with the highest prevalence in the central division of the city (Table 1). The frequency of positive samples from the different geographical parts of the city in the PCR method was higher than that in the flotation method, although the difference was not found to be statistically significant ($\chi^2 = 0.53$, $P_v = 0.464$) (Table 1).

Table 1
Results of the polymerase chain reaction (PCR) and microscopy methods for the detection of *Toxocara* eggs in the soil of five divisions of Ahvaz city.

Area	No. of examined soil samples	Method of diagnosis						
		PCR	%	CI	Microscopy	%	CI	P_v
North	46	11	23.9	12.5–38.7	11	23.9	12.5–38.7	1
West	40	17	42.5	27.04–59.1	15	37.5	22.7–54.1	0.648
East	39	14	35.8	21.2–52.8	12	30.7	17.01–47.6	0.630
Center	43	20	46.5	31.1–62.3	17	39.5	24.9–55.5	0.512
South	42	9	21.4	10.2–36.8	9	21.4	10.2–36.8	1
Sum	210	71	33.8	27.4–40.6	64	30.4	24.3–37.2	0.464

CI, confidence interval.

The kappa index was calculated for overall agreement, that PCR and microscopy results were in agreement (kappa = 0.924, P_v = 000).

The number of *Toxocara* eggs in the soil samples varied from 1.0 to 13.0 per 200 g. A study of embryonated eggs recovered by the flotation method revealed that one or more embryonated eggs existed in 16 samples. There was a significant difference ($p < 0.05$) in the embryonation rate among the different areas examined, with the highest rate of fully embryonated eggs observed in the streets (3.8%) (Fig. 1). Animal feces were found in 51 of 210 (24.3%) samples. No significant difference was found in the proportion of the presence of feces in the soil and contamination rate.

Of the different sampling areas of the city including squares, streets, public parks and rubbish dumps, a slightly higher prevalence of soil contamination was determined by the PCR method (33.8%); however, there was no statistically significant difference between the microscopy and PCR methods ($\chi^2 = 1.171$, $P_v = 0.279$) (Table 2). Using species-specific primers, the PCR method showed 380 bp fragments from *T. canis* and 370 bp fragments from *T. cati*, while no amplification was observed in the negative controls (Fig. 2). The PCR method showed that 59 (28%) and 12 (5.7%) of samples contained eggs of *T. cati* and *T. canis*, respectively, and no mixed contamination was observed. Multiple sequence alignment of nine randomly selected samples confirmed the PCR results; the results revealed conserved nucleotides among the ITS2 sequences obtained and high sequence homology with a small number of differences, corresponding to punctual base substitution. Phylogenetic trees were obtained; the values of 75% and above in the bootstrap test of phylogenetic accuracy indicate reliable grouping among the different *Toxocara* sp. (Fig. 3).

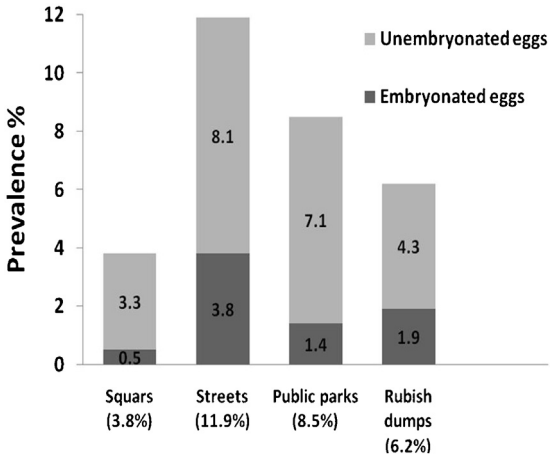

Fig. 1. Prevalence of *Toxocara* eggs in the soil of squares, streets, public parks, and rubbish dumps recovered by the flotation method.

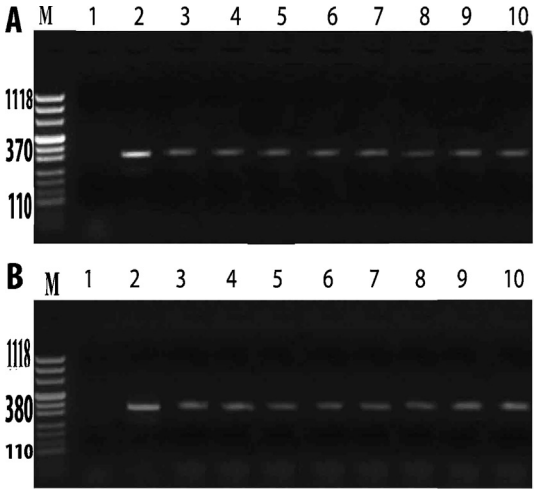

Fig. 2. DNA amplification of *Toxocara* eggs by polymerase chain reaction (PCR) on a 1.8% agarose gel: (A) lane M, marker; lane 1, negative control; lane 2, *T. cati* positive control 370 bp; lanes 3–10, *T. cati* recovered from soil; (B) lane M, marker; lane 1, negative control; lane 2, *T. canis* positive control 380 bp; lanes 3–10, *T. canis* recovered from soil.

4. Discussion

The present study investigated the prevalence of *Toxocara* eggs in soil samples collected from Ahvaz city, southwest Iran, using the flotation and molecular methods. Depending on climate and seasonal variations worldwide prevalence of *Toxocara* eggs in the soil of public places, especially in public parks, was estimated to be 9.75%, 6.73%, 28.31%, 11.57%, 0.55%, 14.03%, and 11.87% in North America, Latin America, Europe, Asia, Middle East, Australia, and Turkey, respectively (Avcioglu and Balkaya, 2011).

Using the nitrate flotation method, 30.4% of the soil was found to be contaminated with *Toxocara* eggs, which was higher than in similar studies previously conducted in various areas of Iran. Epidemiological studies conducted in Iran have documented the contamination rate of public places as 6.3% in Shiraz city in the south (Motazedian et al., 2006) and 22.2% in Khorramabad city in

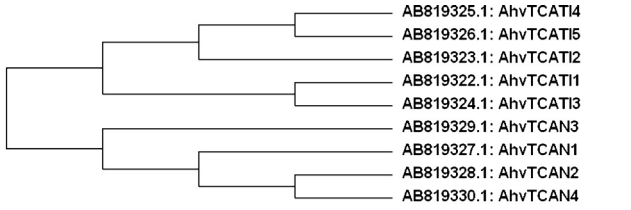

Fig. 3. Dendrogram obtained for *Toxocara* spp. isolates/genotypes by the maximum likelihood analysis at the ITS2 gene. The result strongly supports the placement of *T. cati* and *T. canis* in distinct species, although they are supposedly an anamorph/teleomorph pair.

Table 2
Comparison of methods for the detection of *Toxocara* sp. eggs detected using microscopy and polymerase chain reaction (PCR) in different sampling areas.

Site	No. of examined soil samples	PCR			Microscopy			
		np	*p*	CI	np	*p*	CI	P_v
Square	33	9	4.3	13.2–45.5	8	3.8	11.09–42.25	0.77
Streets	91	29	13.8	22.5–42.5	25	11.9	18.6–37.8	0.51
Public parks	65	20	9.5	19.9–43.4	18	8.5	17.3–40.2	0.70
Rubbish dumps	21	13	6.2	38.4–81.9	13	6.2	38.4–81.9	1
Sum	210	71	33.8	27.4–40.6	64	30.4	24.3–37.2	0.464

np, number positive; *p*, prevalence (positive %); CI, confidence interval.

the west (Zibaei et al., 2010). These results may be due to several factors such as climate, poor sanitation, cultural and social conditions, soil texture, and the presence of dogs and cats, especially the number of stray cats and dogs. Analysis of the results from the geographical regions of the city allowed us to ascertain the profile of *Toxocara* soil contamination distribution among different parts of Ahvaz. The lack of fencing in public parks and the free access to stray animals in the places that were studied may be some of the risk factors for soil contamination. A previous study conducted in Turkey has demonstrated a difference between soil contamination rates in fenced and non-fenced parks (Avcioglu and Balkaya, 2011).

The relationship of soil contamination and cultural and socioeconomic levels has also been previously shown (Capuano and Rocha, 2005); interestingly, our results showed the lowest prevalence of contamination in the southern parts of the city (9%), represented by the low socioeconomic levels of inhabitants, a poor area with a growing number of stray animals.

Mizgajska showed that the prevalence of toxocariasis in humans is related to the degree of soil contamination by *Toxocara* eggs (Mizgajska, 2001), because *Toxocara* eggs are extremely resistant to environmental conditions (Glickman and Shofer, 1987), and may be infective for a long time. The causative agent of human toxocariasis is infection by embryonated eggs and our study showed that 7.6% of the eggs in the soil samples were embryonated. It seems that the extent of humidity in the soil as well as the temperature strongly influences the embryonation of eggs (Stojčević et al., 2010). Our study was conducted in the rainy season, when average temperatures are between 15 and 25 °C from November to February in southwestern Iran. Previous studies have confirmed that the optimal temperature for *Toxocara* egg development in the soil ranges from 23 to 30 °C (Rocha et al., 2011).

The differentiation of *Toxocara* spp. is important in epidemiological and serological studies. Lee et al. (2010) proposed that in human toxocarosis, *T. cati* may be more important than *T. canis* (Lee et al., 2010). Fisher (2003) showed that in comparison with *T. canis*, the zoonotic potential of *T. cati* is underestimated and emphasized, "there is a need for an increased understanding of the epidemiology of infection in cats because in many areas assumptions are based on the epidemiology of *T. canis* infection in dogs" (Fisher, 2003). Few studies have been conducted to differentiate of eggs of *T. cati* and *T. canis*; however, these few studies have shown that *T. cati* has contributed considerably to the level of infection (Uga et al., 1989).

Microscopic observations based on the morphological features of *Toxocara* eggs are inadequate, especially in eggs isolated from the soil. Molecular methods for the discrimination of *Toxocara* spp. from soil samples have been previously performed (Borecka, 2004; Borecka and Gawor, 2008; Fogt-Wyrwas et al., 2007). As soil contains several inhibitors of PCR, to overcome this limitation, molecular analyses were performed after the flotation method because washing eggs several times is important for the efficiency of DNA detection (Fogt-Wyrwas et al., 2007). Fogt-Wyrwas et al. (2007) recommended the PCR method, as it confirms the results of microscopic findings during the routine examination of soil samples.

In the present study, the ratio of *T. canis to T. cati* was found to be 1:5 by the molecular method, which is in accordance with similar reports and shows that cats are the main contaminating animals (Matsuo and Nakashio, 2005; Uga et al., 1996). In the city, the population of stray dogs is less than cats, and increasing number of stray cats and the absence of dogs as pets, because of the Muslim culture, are the main reasons of our findings. Also sequencing and phylogenetic studies of the ITS2 gene presented here confirmed our findings on presence of two different *Toxocara* spp. BLAST analysis revealed significant homology between our DNA sequence data and *Toxocara* spp. sequences previously submitted to the GenBank with accession numbers AB743607.1, AB743609.1, AB743611.1, AB743613.1, for *T. cati* and AB743614.1, AB743615.1, AB743616.1, AB743617.1 for *T. canis* (Khademvatan et al., 2013). Our results are in agreement with previous studies by Uga et al. (1989) and Shimizu (1993), who used scanning electron microscopy on *Toxocara* eggs recovered from the sandpits of public parks and reported *T. canis*:*T. cati* ratios of 1:3 and 2:3, respectively (Shimizu, 1993; Uga et al., 1989).

In conclusion, this study is the first to report the soil contamination by *Toxocara* eggs in different areas of Ahvaz city, southwestern Iran. The information about the rate of environmental contamination will help the local health systems develop effective prevention and control, such as controlling stray cats, promoting laws to protect public areas and enlightening citizens to the importance of preventing defecation by cats and dogs in public areas.

Although in our study the PCR method showed similar results to the conventional microscopy method and was not statistically different, the identification process was easier. The molecular method appeared to be a sensitive and reliable tool for detection and discrimination of *Toxocara* spp. in soil samples, a result that might possibly be overlooked when conducting a microscopic examination.

Conflict of interest

The authors declare no conflict of interests.

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